International Journal of Agricultural
Science and Research (IJASR)
ISSN(P): 2250-0057; ISSN(E): 2321-0087
Vol. 4, Issue 4, Aug 2014, 77-86
© TJPRC Pvt. Ltd.

EFFICIENCY OF RAPD, SSR AND ISSR MARKERS IN EVALUATING THE GENETIC FIDELITY FOR MICROPROPOGATED *MUSA ACCUMINATA* PLANT EXPOSED TO COAL EXTRACTED HUMIC ACID AND COMMERCIALLY AVAILABLE PRODUCTS

S. DHANAPAL[1], D. SATHISH SEKAR[2] & P. MANASA SATHEESH[3]

[1]Research Scholar, Department of Biotechnology, St. Peter's Institute of Higher Education and Research, St. Peter's University, Tamil Nadu, India

[2]Assistant Professor and Head, Department of Biotechnology, Arignar Anna College Arts and Science, Krishnagiri, Tamil Nadu, India

[3]Director, Genewin Biotech, Hosur, Krishnagiri, Tamil Nadu, India

ABSTRACT

Genetic variations, if any, in *Musa accuminata* exposed to Humic acid ; Coal extracted Humic acid (CHA), Biochar Humic acid (BHA) and Commercially available Keradix (KHA) was evaluated using Random amplified polymorphic DNA (RAPD), Simple Sequence Repeat (SSR) and inter simple sequence repeat (ISSR). In this study tissue culture of *Musa accuminata* enhanced with various concentrations ranging from 0.1 – 0.5% of Humic acid was carried out in MS medium. The isolated DNA amplified with 2 ISSR, 2 SSR and 2 RAPD primers. The PCR based markers such as RAPD and ISSR were useful techniques for detection of variation atgenetic level. The present protocol used for large scale production of Humic acid enhanced *Musa accuminata* produce genetically stable plants and was proved that it can be used without any problem.

KEYWORDS: Genetic Fidelity, Humic Acid (HA), Keradix, *Musa Accuminata*, Inter-Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR)

INTRODUCTION

Banana (Musa spp.) is the fourth most important food crop in the world as well as in India (Ganapathi et al, 1991). *Musa accuminata*which is very popular and with high commercial value, has a high demand on markets due to its sweet aroma, taste and higher post harvest life. However, the shortage of planting material and synchronization of fruit ripening are two major bottlenecks that cause unavoidable trouble to local banana growers. There is thus a need to establish a micropropagation protocol for this banana cultivar. (Olivia Saha Roy et al., 2010).There have been scarce reports on the effect of HA on plant growth and development in vitro propagation, especially at low nutrient level in which the sources of plant food were limited (Kullanart Obsuwan et al., 2011).

The organic matter in soil is divided into that which is partially decomposed matter and that which is fully decomposed, and termed 'humus'. This humus can be further divided into soluble humic acids and insoluble 'humins'. The soluble humic acids have three major divisions 'humic acid', 'ulmic acid' (also called hymatomelanic acid) and 'fulvic acid'. Fulvic acid is a short chain molecule which is yellow in color and soluble. In horticulture it is the humic, and particularly the fulvic acids which are most reactive and effective in stimulating plant growth. (Lynette Morgan, 2012).

Plant growth is influenced indirectly and directly by humic substances. Once the humic acid, enter plant cells several biochemical changes occur in membrane and various cytoplasmic component of plant cells (Robert E. Pettit, 2007). The term humic acid represents a group of powerful natural substance that are so complex that science will not be able to replicate them from generation to come. Humic acids are derived from highly concentrated natural deposits, the most common deposits is leonardite (form of oxidized lignite). The presence of insoluble humic acid can commonly be found in ordinary soil in lower concentration (0.2% to 10%). Once raw leonardite are converted in to water soluble humates all of the humic acid and fulvic acids components will be biologically active and play important role in plant and soil stimulation. Humic acids are not a significant source of plant nutrient but are a soil stimulant and a transportation vehicle for carrying nutrient into plants. (Boris Levinsky, 2001).

The uptake of major plant nutrients is mediated by humic substances. One stimulating effect of humic substance on plant growth is enhanced uptake of major plant nutrients such as Nitrogen (N), phosphorus (P), and potassium (K). When adequate humic substances are present within the soil the requirement for "NPK" fertilizer application is reduced. The best source of humic substances for fertilizer use is from leonardite. Leonardite is defined as highly oxidized low grade lignite that contains a relatively high concentration of smaller molecular units. (Robert E. Pettit, 2007).

The positive effects of humic substances on the growth of plants in Gramineae family has been well documented (Chen and Aviad, 1990). Dormaar in 1975 reported increased N uptake by rough fescue (Festuca scabrella Torr.) in response to application of humic substances extracted from 3 soils, while P, K, Ca, Mg and Na uptake was unaffected. Humic acid have long recognized that play a major role in producing morphological and physiological effects in plants (Nardi. S et al, 2002 and Eyheraguibel et al., 2008).

It has been reported that humic acids are able to stimulate or inhibit plant growth depending on their differences in origin, nature and concentration. The application of humic substances to nutrient solution, to soil or sand has been documented and the results showed that they enhanced significant growth responses (Lulakis M.D and Petsas .S.I, 1995).

Molecular study of plant relies on high quality and yield of DNA obtained. The leaves of the banana contain high level of polysaccharides, polyphenols and secondary metabolite (Shankar K et al., 2011). DNA extraction from young leaves is most commonly preferred compared to mature leaves as content of polyphenols and polysaccharides are less in young leaves (Zhang and Stewart, 2000). A quick, simple and reliable method for DNA extraction is the modification of Doyle and Doyle (1990). It is a CTAB based extraction procedure modified by the NaCl to remove polysaccharides and polyvinylpyrrolidone to eliminate polyphenols during DNA purification. DNA yield from this procedure is high (up to 1mg/g of leaf tissue). (Lodhi and Muhammad A, 1994) and purity ratio was ranging from 1.7 to 1.8 showing minimal level of secondary metabolite contamination (Shankar K, et al, 2011).

Morphological description, physiological supervision, karyotypic analysis, biochemical estimation and field assessment were used to detect any type of genetic variation, but presently molecular markers have complemented over traditional method to detect and monitor the genetic fidelity of tissue culture derived plantlets and variety identification. Molecular markers have been used successfully to determine the degree of relatedness among individuals or group of accession to clarify genetic structures or variation among accession, population, varieties and species (G.R.Rout et al., 2009).

The present study is to study the effect of various concentrations of humic acid in Musa accuminata, a cavendish variety often called as G-9 (Grand Naine) with different strengths of the Murashige and Skoog medium (Murashige. T and Skoog. F, 1962). Numerous publications proved the variations produced by growth regulators in plants Invitro. Since HA is rich in auxins, in combinations with other cytokinins has the possibilities to produce somoclonal variants in micropropagation of plants. Also HA itself has some toxic metals associated with it based on the origin. (Lawrence Mayhew, 2004). Thus the molecular level studies is carried out with the RAPD and ISSR techniques to check the genetic fidelity in HA supplemented *Musa accuminata*.

MATERIALS AND METHODS

Extraction of Humic Acid From Leonardite

Sample Collection

The coal sample (leonardite) was collected from Mines II of Neyveli lignite corporation, Neyveli and the *Musa accuminata* plant tissue cultured at Genewin Biotech, Hosur.

Extraction of Humic Acid with Various Solvents

Humic acids were extracted from the resulting leonardite, using extraction methods that are capable of extracting humic acids. The extraction of humic acid from leonardite 5 g was extracted with 50 ml of (0.1 M NaOH, 0.1 M KOH, 0.1 M $Na_4P_2O_7$, 0.25 M NaOH, 0.25 M KOH, 0.25 M $Na_4P_2O_7$) and stirred for 1 min. The pH of the suspension was maintained at 13 by addition of NaOH (20%, w/v) and allowed to stand for 3 h. The mixture was centrifuged at 3500 rpm for 15 min in order to eliminate the precipitation. The supernatant was then acidified with 50 ml of 0.1 M Hcl and stirred for 1 min. The pH of the suspension was adjusted to 1 by the addition of Hcl (10%, w/v), and it was allowed to stand for overnight. Both fulvic acids (supernatant) and humic acids (precipitation) fraction were obtained by centrifuged at 3500 rpm for 15 min in order to eliminate the supernatant and precipitation washed once with distilled water. The humic samples were dried at 60^{o}C, the highest yield from each solvent extract is weighed and concluded.

Estimation of % Humic Acid

The estimation of the percentage of humic acid was elaborated by Stevenson in 1994. 0.1g of Humic acid was weighed and ground in to a fine powder. It was then dissolved in 10 ml of extraction buffer containing 0.2M NaOH, 0.0032 M DTPA (Diethylene triamine pentaacetic acid, ROLEX-Mumbai), 2% ethanol. Mix the sample well, centrifuge the aliquot of the sample to remove any particulates. The supernatant is saved as the sample; 1 ml of the sample was taken and mixed with 5 ml of water. OD was taken at 450 nm using Titan Biotech Humic acid as standard (50-300mg).

Extraction of DNA from Humic Acid Propagated Plant

One – two grams of humic acid propagated leaf sample was taken.The young leaf samples are cut in to pieces using scissors and ground well in to powder by using liquid nitrogen in a prechilled mortar and pestle. 1% ß mercaptoethanol was added to the extraction buffer and it is warmed at 65 °c for 10 to 15 minutes. 10 -20 ml of warmed extraction buffer was added to the ground sample. The sample was thoroughly mixed to form slurry and transferred to a screw capped 30 ml centrifuge tube. 100-200 mg of polyvinylpyrollidone (PVP) was added The tubes were incubated at 65°c for 30 minutes with occasional mixing and cooled to room temperature.Equal volume of chloroform:octonal (24:1) were added to the slurry The slurry was centrifuged at 6000 rpm for 5 minutes at 4°c. The supernatant was transferred to the fresh tube and second extraction can be performed if the aqueous phase was cloudy

in appearance.0.5 volumes of 5M NaCl was added and mixed well.Equal volume of ice cold absolute alcohol was added and refrigerated for 15 to 20 minutes.The sample was centrifuged at 3000 rpm for 3 minutes and then increased to 5000 rpm for additional 3 minutes at room temperature. This differential spinning will help to keep the DNA at the bottom of the centrifuge.The supernatant was poured off and the pellets were washed in 80% ethanol. The ethanol was evaporated by leaving the tube at 37 °c for 10 to 15 minutes.The pellets were resuspended in 0.5 ml of 1X TE buffer.

RNA Se TREATMENT

2µl of RNAse stock solution was added to the nucleic acid mixture in the eppendorf tube and incubated at 55°C for 10 minutes or 37°C for 1 hour.Equal volume of (0.5ml) phenol: chloroform was added and centrifuged at 10000 rpm for 5 minutesThe upper aqueous layer was collected and transferred in to fresh tube and more than double volume of 100% ethanol and 50 µl of 3M sodium acetate was added.The tubes are kept at -20°C over night for precipitation. In the second day the precipitated sample was centrifuged at 12000 rpm for 15 minutes at 4 °C.The pellets were collected and washed with 0.5 ml of 70% ethanol.The sample was centrifuged at 10000 rpm for 5 minutes at 4 °C.The pellets were re-suspended in 100 ml of 1X TE.The re-suspended pellets were collected and stored at -20 °C.

Spectrometric Estimation of DNA

Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to a concentration of 25 ng/µl before use. To 5 or 10 µl of 1/100 diluted sample in 1x TE buffer, 1.95/1.90 ml 1X TE buffer was added. Take 2ml of the 1X TBE buffer in a quartz cuvette and baseline correction was done. The absorbance was noted at 260 and 280 nm and ratio was calculated. An absorbance of A260 of 1.0 corresponds to 50 µg double stranded DNA /ml of the solution. From the above method, the concentration and Purity of DNA in the test samples was calculated.

RESULTS

Quantification of Humic Acid

The extraction of humic acids from leonardite is done using 5 g with 0.1 M KOH yielded 0.8813 g, 0.25 M K0H yielded 0.3312g, 0.1 M NaOH yielded 0.2216 g and 0.25 M of NaOH yielded 0.2566 g, 0.1 M Na4P2O7 yielded 0.6273g and 0.25 M Na4P2O7 yielded 0.6994g. Results of extractant that used for extracting humic acids from leornardite showed that the greatest yield of humic aicds was obtained in 0.1M KOH and the lowest yield in 0.1M NaOH. So KOH is selected as the extracting solvent for the humic acid extraction process.

Table 1: Estimation of HA

Standards (Mg)	Concentration (Ppm)	Absorbance	Samples	Concentration of the Sample (Ppm)	Absorbance of the Sample
50	50	0.8135	Coal CHA (Leonardite)	6.4703	0.3926
100	100	13645	Humic Rooting (BHA)	271.5272	3.5663
150	150	2.3203	Keradix (KHA)	307.7484	4.0000
200	200	2.9488			
250	250	3.2968			
300	300	3.7192			

Table 2: Final Ppm for Standards

Standards (Mg)	Concentration (Ppm)	Absorbance	Samples	Concentration of the Sample (Ppm)	Dilution Factor (6 X)	Absorbance of the Sample
35	35	0.8135	Coal Leonardite	45.29	271.74	0.3926
70	70	13645	Humic Rooting	190.07	1140.42	3.5663
105	105	2.3203	Keradix	215.435	1292.58	4.0000
140	140	2.9488				
175	175	3.2968				
210	210	3.7192				

Since the samples were of 70%, the standards concentration must be converted to 70% from 100%. The standard concentrations for 100% were 50, 100, 150, 200, 250, 300 mg respectively. For 70%, the standard concentrations were converted as 35, 70, 105, 140, 175, 210 mg respectively.

The dilution factor for the estimation of Humic acid was 6 X. For 1 ml of the sample, they were diluted 5 times as the sample concentration was high. So, the Dilution factor was 6X (1 X + 5X). The concentration of the samples with dilution factor was mentioned in the above table.

Isolation of DNA

Genomic DNA was isolated from leaf tissue of Humic acid and commercially available Humic acid incorporated *Musa accuminata*plants. The quality and quantity of DNA was checked by agarose gel electrophoresis. The final DNA concentration of each sample was found to be 6. 520 µg/ml for BHA, 5.982 µg/ml for CHA and 6.253 µg/mlfor KHA.

DNA Amplification

RAPD markers are oligonucleotide fragments used for PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequences, which are able to differentiate between genetically distinct individuals. The RAPD primers are random 10mer nucleotide sequence, which can bind to any part of the genomic DNA during PCR amplification.Another technique called Inter Simple Sequence Repeat (ISSR) reveal numerous microsatellite regions by using primers that may be anchored with one or two nucleotides on either the 5' or 3' end of a repeat region and extend into the flanking region.

Initially genomic DNA of the GW04 NA and GW04 HU were extracted from young leaves using modified CTAB method. Optimum PCR conditions for RAPD, SSR and ISSR were standardized with various quantities of template DNA (12.5, 25 and 50 ng), dNTPs (100, 200 and 300 µM) and MgCl2 (0, 1, 2 and 3 mM). Later, RAPD amplifications were performed routinely using PCR mixture (25 µl) which contained 25 ng of genomic DNA as template, 1x PCR buffer (Himedia, Mumbai), 200 µM dNTPs (Merck, Bangalore), 1 unit (U) of taq polymerase (Merck, Mumbai), 1 µM of each primer with various concentrations of MgCl2 (Himedia, Mumbai) depending on the primer (Merck, Mumbai. PCR was performed at initial denaturation at 93°C for 4 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 36°C and 2 min extension at 72°C with a final extension of 72°C for 10 min using a thermal cycler (Techne thermocycler).

For SSR and ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. PCR mixture (25 µl) which contained 25 ng of genomic DNA as template, 1X PCR buffer

(Himedia, Mumbai), 200 µM dNTPs, 1 Unit (U) of Taq polymerase, 1µM of each primer with various concentrations of MgCl2 depending on the primer. PCR was performed at initial denaturation of 94°C, 1 min annealing at 2°C lower than melting point for each primer and 2 min extension at 72°C with a final extension of 72°C fro 10 min using a thermal cycle (Techne). Amplified products were resolved by electrophoresis on 1.4% agarose gel (Bhatia et al, 2010). Primers are selected with the following references.

- RAPD PRIMER- Venkatachalam Lakshmanan et al, 20072.
- ISSR PRIMER- Venkatachalam Lakshmanan et al, 2007 and H.P.Singh et al, 20113.
- SSR PRIMER- Lorenna Alves Mattos et al, 2010

Table 3: List of ISSR, SSR and RAPD Primers Used for Genetic Fidelity Testing

Primer	Primer Sequence (5'-3')	Length
RAPD OPM-16	GTAACCAGCC	10
RAPD OPM-20	AGGTCTTGGG	10
ISSR UBC-834	$(AG)_8$ YT	18
ISSR UBC 836	$(AG)_8$ YA	18
SSR AGMI 67-68	ATACCTTCTCCCGTTCTTCTTC/ TGGAAACCCAATCATTGATC	22/21
SSR AGMI 103-103	CAGAATCGCTAACCCTATCCTCA/ CCCTTTGCGTGCCCCTAA	23/18

Data Analysis

For RAPD, SSR and ISSR profiles, the well, resolved and consistently reproducible fragments ranging from 200 bp to 2.0 kb were scored as present or absent. For detecting any genetic change, all the RAPD, SSR and ISSR results were compared with each other for all the DNA samples.

RAPD

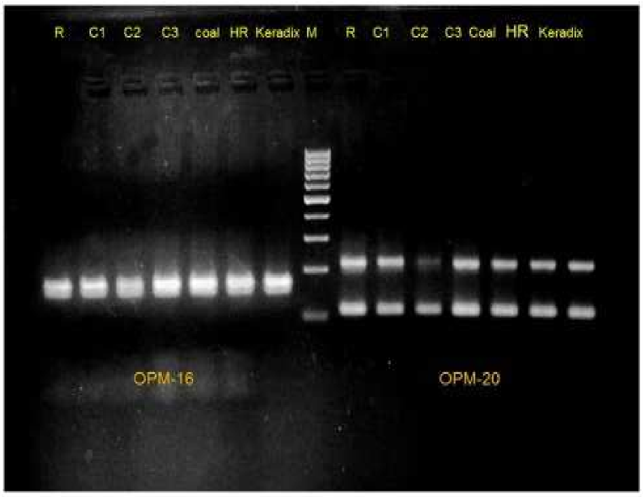

Figure 1: Genetic Fidelity Testing of Native, Coal Extracted and Commercially Available Humic Acid Propagated Plants Using Rapd OPM 16 and OPM 20

Figure 2: SSR Amplification Pattern Obtained for Control Plants with BHA, CHA and KHA Exposed Shoot Cultures Generated by AGMI 67/68, AGMI 103/103 Primers. Himedia 1 Kb Ladder

Figure 3: ISSR Amplification Pattern Obtained for Control Plants with BHA, CHA and KHA Exposed Shoot Cultures Generated by ISSR UBC 836 Primer. Himedia 1 Kb Ladder

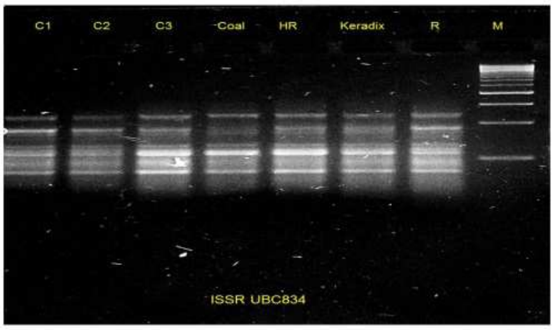

Figure 4: ISSR Amplification Pattern Obtained for Control Plants with BHA, CHA and KHA Exposed Shoot Cultures Generated by ISSR UBC 834 Primer. Himedia 1 Kb Ladder

Note

'R' refers to reference plant belonging to another species.

Table 4: Details of Primers Used to Detect Variation in HA Enhanced Plants and its Amplified Products Using Alphaease Software

Primer Number	No. of Bands Amplified	No. of Monomorphic Bands	No. of Polymorphic Bands	Size of Band Amplified
RAPD OPM-16	1	7	0	110 – 308 bp
RAPD OPM-20	2	14	0	225 - 298 bp
ISSR UBC-834	7	49	0	54 – 294 bp
ISSR UBC 836	6	42	0	45 - 356 bp
SSR AGMI 103-103	1	7	0	140- 210 bp
SSR AGMI 67-68	1	7	0	148 – 224 bp

DISCUSSIONS

True to the type clonal fidelity is very important criteria in the mass multiplication of any plant Invitro. A major problem encountered with the Invitro culture is the presence of somaclonal variations amongst the subclones due to the over multiplication, due to growth hormone concentration or environmental stress. Care must be taken not to induce the possibility in emerging of the somoclonal variants in trials that would affect the whole micropropagation systems.Use of two marker systems i.e., RAPD and ISSR would help in amplifying different part of the genome. The RAPD primer binds randomly in any part of the genome and amplifies it as the length of the primers is also short (10mer). Whereas, the ISSR markers binds only in between the simple sequence repeats and amplify. The binding of ISSR primers is more precise as the number of nucleotide is more as compared to RAPD. Along with the primer size and the higher annealing temperature of the ISSR primers (50^{o}C) as compared to RAPD (45^{o}C) used in the present study, the binding of the primers stringency increase and hence produces more stable bands. It was observed in our studies that the amplified products of RAPD OPM -16 showed a amplified band size of 110 – 308 bp and RAPD OPM -20 showed a amplified band size of 225 – 298 bp , whereas, ISSR primers amplified bands in the range of 54 – 294 for 1SSR UBC-834 and 45 - 356 bp for ISR UBC - 836 . It was also observed from the studies that, use of more number of markers would yield more amounts of details of the genome and chances of detection of any variation.The RAPD primers RAPD OPM – 16 and RAPD OPM-20 gave 2, 1 bands respectively the number of bands and molecular weight for each band amplified by every primer was same this also show there was no polymorphism.SSR AGMI 103-103 and SSR AGMI 67-68 gave 1 amplified band and 7 monomorphic bands with size ranging from 140 – 210 bp and 148 – 224 bp.ISSR primer UBC-834, BC 836 amplified six bands from the genomic DNA isolated from all control and Humic acid propogated plants. Humic acid is the new natural stimulant used in this trial to study the efficacy in micropropagation. RAPD, SSR and ISSR were carried out with selective primers where there is no variations in the bands detected which is a key element for the safety use of Humic acid in the plant tissue culture.

CONCLUSIONS

To study the genetic fidelity in *Musa accuminata* control plants against the HA (CHA, BHA, KHA) exposed plants, we adopted the use of the three PCR based techniques, RAPD, SSR and ISSR for the identification of somaclonal variation of in Grand Naine because of their simplicity and cost effectiveness. This study screened a large number of random primers that are common for higher plants. Since there were no changes in the banding pattern observed in HA cultured plants and control plants (Native), we conclude that the Humic acid can be considerably used safely in the tissue culture of Musa accuminata without much risk of genetic instability.

ACKNOWLEDGEMENTS

The authors record their sincere thanks to the Management and Dr. M. Subbiah, Pricipal of Aringar Anna College (Arts and Science), Krishnagiri, Tamilnadu, India for their unstinting support rendered towards us. We also extend our gratitude to Dr.V.Ravikumar, Assistant professor, Department of Biochemistry, Bharathidhasan University, Tiruchy for his valuable suggestions throughout this research work. Last but not least we thank Dr. V. Palani, Managing Director, Genewin Biotech, Hosur for providing necessary laboratory facilities to carry out this work in their DBT certified laboratory.

REFERENCES

1. Boris V. Levinsky, (2002). Research Update on fulvic acids-Are there really naturally occurring fulvic acids? 0n Tera Vita. http://www.teravita.com/Technical%20Articles/fulvic%20acids.htm.
2. Chen Y, Aviad T (1990). Effects of humic substances on plant growth. In: MacCarthy P, Clapp CE, Malcolm RL, Bloom PR, editors. Humic Substances in Soil and Crop Science: Selected Readings. Madison, WI: American Society of Agronomy Inc., Soil Science of America, Inc. pp. 161–186.
3. Dormaar J. F (1975). Effects of humic substances from Chernozemic Ah horizons on nutrient uptake by Pfta seolus vulgaris an (l Festuca scabrelLa. can. J. Soil Sci. 55: I I 1-l 18.
4. Doyle, J. J. and J. L. Doyle(1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.
5. Eyheraguibel B, Silvestre J, Morard P (2008). Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize. Bioresource Technology 99, pp. 4206-4212.
6. G. R. Rout and S. K. Senapati (2009). Studies on genetic identification and genetic fidelity of cultivated banana using ISSR marker, Southern cross journal 2(6), pp 250-258.
7. H. P. Singh, S. Uma, R. Selvarajan and J.L. Karihaloo (2011), Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), Asia-Pacific Association of Agricultural Research Institutions (APAARI), New Delhi.
8. K. Shankar, L. Chavan (2011), An improved DNA extraction protocol from invitro banana cultivator. Asian Journal of biotech vol: 3(1), pp 84-90.
9. Kullanart Obsuwan, Suluck Namchote, Natdhera Sanmanee, Kamolchanok Panishkan and Sirichai Dharmvanij (2011). Effect of various concentrations of Humic acid on growth and development of Eggplant seedlings in tissue cultures at low nutrient level. World academy of science, engineering and technology, Pp.276-278.
10. Lawrence Mayhew Humic Substances as Agronomic Inputs in Biological Agricultural Systems: a Review http://www.fertitech.com/
11. Lodhi, Muhammad A (1994), A simple and efficient method for DNA extraction from grape vine cultivators, vitis species and ampelopsis. plant molecular biology reporter 12(1):6-13.
12. Lorenna Alves Mattos, Edson Perito Amorim, Vanusia Batista de Oliveira Amorim, Kelly de Oliveira Cohen (2010), Agronomical and molecular characterization of banana germplasm, Pesq. agropec. bras, Brasília, v.45, n.2, p.146-154.
13. Lynette Morgan (2011). Growing Green for Australia Maximum Yield- Indoor Gardening- the Pithium Predator.
14. M.D. Lulakis and S.I Petsas (1995). Effect of humic substances from vine canes mature compost on tomato seedling growth, Biosource technology, Vol. 54. Pp:179-182.
15. Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassoys with tobacoo tissue culture. Physical Pl., 15: 473–97.

16. Olivia Saha Roy, Pranay Bantawa, Swapan Kumar Ghosh, Jaime A, Teixeira da Silva, Partha DebGhosh and Tapan Kumar Mondal (2011). Micropropagation and Field Performance of Malbhog: Popular Banana Cultivar with High keeping quality of North East India. In: Tree and Forestry Science and Biotechnology. Global science books.

17. Pettit, Dr. Robert E (2007). Emeritus Associate Professor Texas A & M University, Organic Matter, Humus, Humates, Humic Acid, Fulvic Acid and Humin: for

http://www.montmorillonite.info/Humus%20Humates%20and%20Humic%20Acid.pdf .

18. Serenella Nardi, Diego Pizzeghello, Adele Muscolo and Angelo Vianello (2002). Physiological effects of humic substances on higher plants. Soil Biology & Biochemistry 34, pp. 1527–1536.

19. Stevenson, F (1994). Biochemistry of the formation of humic substances. p. 188-211. *In:* Humus chemistry: genesis, compositions, reactions. John Wiley and Sons. New York, NY.

20. T. R. Ganapathi, P.Suprasanna, V. A. Bapat, V. M. Kulkarni and P. S. Rao (1999). Somatic embryogenesis and plant regeneration from male flower buds in banana. Current science volume 76. No. 9. pp 1228-1331.

21. Venkatachalam Lakshmanan, Sreedhar Reddampalli Venkataramareddy and Bhagyalakshmi Neelwarne (2007), Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers, Electronic Journal of Biotechnology ISSN: 0717-3458, Vol.10 No.1.

22. Zhang and Stewart (2000), Economical and rapid method for extracting cotton genomic DNA, The Journal of cotton science 4: 193: 201.